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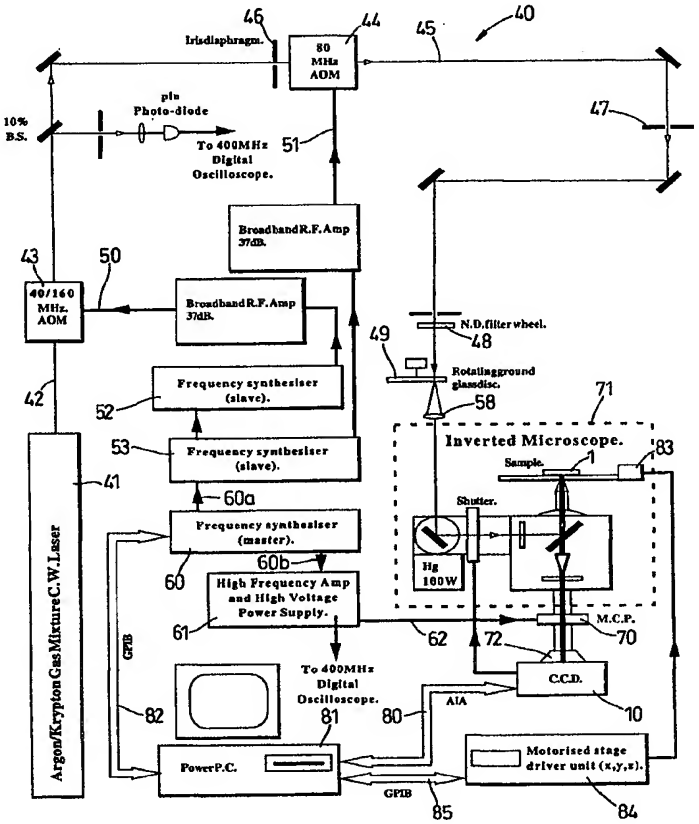
(54) Title: MULTIPLE FREQUENCY FLUORESCENCE LIFETIME IMAGING

(57) Abstract

The application of a high frequency periodic bias voltage to the photocathode of an image intensifier device results in a repetitive modulation of the gain characteristics of the intensifier. By controlling the bias or DC component of the bias voltage it is possible to control the duration of the 'on' state of the device relative to its 'off' state. The amplitude of the higher harmonic content in the square waveform can thus be controlled. In this mode the image intensifier can be used as a mixing device for the simultaneous homodyne detection at each of the harmonics. Applied in a fluorescence lifetime imaging microscopy (FLIM) application, fluorescence lifetime images can be obtained at multiple frequencies in a harmonic set calculated from a single series of phase dependent images. Both the phase and modulation parameters necessary for spatially resolved heterogeneous lifetime calculations are extracted by Fourier analysis of the series of phase dependent images.



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MULTIPLE FREQUENCY FLUORESCENCE LIFETIME IMAGING

The present invention relates to methods and apparatus for determining true fluorescence lifetimes in fluorescent materials, and in particular to a method and apparatus for imaging spatial variations in such fluorescence lifetimes in samples having a composition of varying lifetime species, or species in a multiple of lifetime states, distributed throughout the sample.

Temporally resolved spectra offer a wealth of information about the state of a fluorescent molecule and its immediate environment. The fluorescence lifetime of a molecule is inversely proportional to the sum of all the deactivation pathways out of the excited state. Consequently the fluorescence lifetime of a fluorophore is sensitive to environmental conditions such as pH, ionic strength and hydrophobicity, and to excited state reactions such as fluorescence resonance energy transfer (FRET), molecular quenching and triplet formation. Furthermore, unlike fluorescence intensity measurements, the measurement of fluorescence lifetime is independent of fluorophore concentration and light path length.

In recent years, fluorescence lifetime measurements have been applied in the microscope for elucidating many aspects of cell physiology, where the highly heterogeneous nature of the cell environment makes the interpretation of fluorescence intensity especially problematic.

Fluorescence Lifetime Imaging Microscopy (FLIM) enables the temporal attributes of fluorescence emission to be simultaneously measured in every pixel of a microscope image and has been described in the prior art applied in both the time and frequency domain.

In the prior art, frequency domain lifetime measurements in general employ a repetitively modulated light source (typically a sinusoidally modulated laser source) to excite the fluorophore of interest. The resulting fluorescence emitted by the sample is also modulated at the same frequency but phase shifted and demodulated relative to the excitation source.

From both the phase shift and demodulation parameters the phase (τ_ϕ) and modulation (τ_M) fluorescence lifetimes can be independently calculated. Only in fluorescent samples composed of a single homogeneous species are these quantities equal to each other and to the true fluorescent lifetime of the fluorophore. For composite samples the true fluorescence lifetimes can only be determined from phase shift and demodulation measurements acquired at a number of modulation frequencies. A sample composed of N lifetime species requires the phase shift and demodulation to be measured at a minimum of N frequencies. By fitting these to a pair of dispersion relationships the true fluorescence lifetime composition of the sample can be estimated. Ideally, the circular frequencies of the modulations should be chosen so as to span all the reciprocal lifetimes of the sample. At the high frequencies required for nanosecond fluorescence lifetime measurements (10's-100's of MHz), accurate and precise determination of the repetitive fluorescence waveform can be obtained by the application of heterodyne / homodyne detection techniques. This type of approach has been extensively applied to the lifetime determination of solutions using point detectors i.e. lifetime fluorimeters. Homodyne/heterodyne detection using a point detector can be achieved by mixing the detected fluorescent signal with an electronic mixing signal generated either in a lock-in amplifier or by mixing with the modulated gain characteristics of the detector directly. Typically, such instruments sequentially record phase and modulation data at a number of frequencies.

In prior art fluorescence lifetime imaging techniques, lifetime determination at every pixel of an image is made possible by the use of image intensifiers as frequency mixing devices. An exemplary prior art apparatus and method are described in connection with Figures 1a and 1b, where figure 1b specifically exemplifies the application of the homodyne mixing technique.

A fluorescent sample 1 is irradiated with excitation energy 2 which is intensity modulated at a certain frequency by an acousto-optic modulator 3 driven by the amplified voltage output from a frequency synthesizer 4a. Fluorescence emission 5 (expressed as F(t) in Figure 1b) from the irradiated sample 1 is separated from the excitation light via a dichroic mirror 12 and an emission filter 13 and is focused onto the photocathode 6 of an image intensifier having a microchannel plate device 7. Photoelectrons generated by the light image incident upon the photocathode surface 6 are amplified by electron cascade across the microchannel plate 7, maintaining the spatial resolution of the image. The amplified electron image 8 exiting the microchannel plate strikes a phosphor screen 9 to generate an amplified light image which can be recorded by imaging onto a CCD camera 10.

A second frequency synthesiser 4b, phase-locked to the first and with controllable phase ϕ_G, is used to modulate the gain of the image intensifier (expressed as G(t) in Figure 1b) either by applying the amplified modulated voltage signal 11 across the microchannel plate 7 or (as exemplified in figure 1b) at the photocathode 6. Frequency mixing of the resulting modulated gain characteristics with the fluorescence signal is thus performed at every pixel of the image output 8 (expressed as D(t) in Figure 1b). The phosphor screen 9 of the image intensifier behaves as a low pass

frequency filter and only the low frequency difference signals are observed by the CCD 10 (expressed as $D_{LP}(\phi_G)$ in Figure 1b). Where the frequency content in the gain modulation G(t) matches that in the fluorescence signal F(t), homodyne frequency mixing results in a phase dependent output image $D_{LP}(\phi_G)$ which contains the phase ϕ_F and amplitude M_F of the fluorescence signal. The phase dependent signal $D_{LP}(\phi_G)$ at every pixel of the image is sampled over a full phase cycle ($0° \leq \phi_G \leq 360°$) by recording an image at each phase setting ϕ_G. From a Fourier analysis of the set of phase dependent images $D_{LP}(\phi_G)$ both the phase shift and demodulation associated with the fluorescence signal can be calculated. The minimum number of phase samples is chosen to satisfy the Nyquist criterion.

With prior art FLIM instruments, phase shift and demodulation measurements have only been performed at a single modulation frequency for any one experiment, since sequentially collecting lifetime images at every frequency is prohibitively costly, both in terms of data collection times and in terms of the total time the microscopic sample is exposed to the excitation light. Both these points are especially critical in relation to microscopic measurements since the samples under observation are often dynamic in nature (ie. the movement of fluorescently labelled proteins within the spatial environment of a living cell) and far more sensitive to the effects of photobleaching than cuvette samples, where large sample volumes and diffusion ensure that the fluorophores at the point of illumination are continuously replaced. A further consideration is the huge volumes of data to be processed from multiple sets of phase dependent images.

It is an object of the present invention to provide a method and apparatus for the simultaneous collection of fluorescence lifetime data and images at multiple frequencies.

It is a further object of the present invention to provide a method and apparatus which enables phase shift and demodulation images at multiple harmonic frequencies to be calculated from a single cycle of phase dependent images to thereby reduce sample exposure times. The methods described herein are equally applicable to the simultaneous collection at multiple frequencies of fluorescence lifetime data from cuvette samples using a modulatable point detector ie. by modulating the photocathode voltage of a photo-multiplier tube.

According to one aspect, the present invention provides a method for making fluorescence lifetime measurements comprising the steps of:

irradiating a fluorescent material with a beam of excitation energy intensity modulated with a plurality of frequencies in a harmonic series including a fundamental frequency and at least one harmonic thereof;

receiving a fluorescence emission from said fluorescent material into a detector having a controllable gain and an output;

modulating the output of said detector using said controllable gain, with a modulation function having frequency components corresponding to at least two of said harmonic series of said irradiating beam, and having a controllable phase angle relationship thereto; and

sampling said detector output to determine an amplitude for each of a plurality of phase angles.

According to another aspect, the present invention provides a method of imaging spatial variations in fluorescence lifetimes comprising the steps of:

irradiating a fluorescent material with a beam of excitation energy intensity modulated with a plurality of frequencies in a harmonic series including a fundamental frequency and at least one harmonic thereof;

imaging a fluorescence emission from said fluorescent material onto a detector having a controllable gain and an output;

modulating the output of said detector using said controllable gain, with a modulation function having frequency components corresponding to at least two of said harmonic series of said irradiating beam, and having a controllable phase angle relationship thereto; and

generating an image of spatial variation in the modulated detector output for each of a plurality of phase angles between said excitation beam and said modulation signal.

According to another aspect, the present invention provides apparatus for making multi-frequency fluorescence lifetime measurements comprising:

a source of electromagnetic radiation for irradiating a fluorescent material with a beam of excitation energy intensity modulated with a plurality of frequencies in a harmonic series including a fundamental frequency and at least one harmonic thereof;

means for receiving a fluorescence emission from said fluorescent material into a detector having a controllable gain and an output;

means for modulating the output of said detector using said controllable gain, with a modulation function having frequency components corresponding to at least two of said harmonic series of said irradiating beam, and having a controllable phase angle relationship thereto; and

means for sampling said detector output to determine an amplitude for each of a plurality of phase angles.

According to another aspect, the present invention provides apparatus for imaging spatial variations in fluorescence lifetimes in a sample, comprising:

a source of electromagnetic radiation for irradiating a fluorescent material with a beam of excitation energy intensity modulated with a plurality of frequencies in a harmonic series including a fundamental frequency and at least one harmonic thereof;

means for imaging a fluorescence emission from said fluorescent material onto a detector having a controllable gain and an output;

means for modulating the output of said detector using said controllable gain, with a modulation function having frequency components corresponding to at least two of said harmonic series of said irradiating beam, and having a controllable phase angle relationship thereto; and

means for generating an image of spatial variation in the modulated detector output for each of a plurality of phase angles between said excitation beam and said modulation signal.

Embodiments of the present invention will now be described by way of example, and with reference to the accompanying drawings in which:

Figure 1a shows essential components of a prior art phase sensitive homodyne imaging system and Figure 1b illustrates the principles thereof;

Figure 2 shows a graph (Fig 2b) plotting the photoelectron transfer characteristics, in response to constant illumination, of an image intensifier device (Fig. 2a); the effective gain response of the image intensifier to a sinusoidal photocathode voltage (Fig. 2c) is given in Fig. 2d, which can be modelled by a square pulse function with adjustable width D (Fig. 2e).

Figure 3 shows a series of plots (3a to 3e) illustrating the variation in the relative modulation depth (the ratio of the amplitude in the gain at harmonic n to the average gain, G_n/G_0) against the fractional pulse width

for each of the first five harmonics, calculated from the square pulse wave model gain function shown in Fig. 2e;

Figure 4 shows an exemplary apparatus for carrying out the method of the present invention;

Figure 5 shows a series of graphs of experimental data plotting the average gain G_0 and relative modulation (G_n/G_0) for the first four harmonic terms in the gain response of an image intensifier measured as a function of the relative photocathode voltage bias;

Figure 6 shows the harmonic content of phase sampled reflected excitation light for a set of 'low' and 'high' frequency modulations; and

Figure 7 shows the results of processing mfFLIM data in order to separate the fluorescence of two green fluorescent protein mutants co-expressed in a live Hela cell.

Figure 1 has already been described in detail, in connection with the prior art. Where features of the present invention have corresponding or similar function to the features of Figure 1a, the same reference numerals will be used.

The present invention takes particular advantage of the photoelectron transfer characteristics of an image intensifier device in order to enable control of the higher harmonic content of an image output of the intensifier, by square pulse wave modulation of the gain characteristics.

It has been recognised that, because the application of a positive voltage applied to the photocathode of an image intensifier acts as a switch to the flow of photoelectrons, the application of a zero mean sinusoidal voltage results in a square wave modulation of the gain characteristics of the device as the sinusoidal voltage alternates in polarity. By controlling the bias or

DC component of the sinusoidal voltage, it is possible to control the duration of the 'on' state of the device relative to its 'off' state.

In this way the amplitude of the higher harmonic content can be controlled from the width of the square pulse waveform produced. Relative modulation depths (the ratio of the amplitude in the gain for the *n*th harmonic component G_n to the average gain G_0) of up to 400% are possible from this form of modulation. In this mode each of the harmonics in the gain of the image intensifier can be used in the simultaneous homodyne detection of matching harmonic modulations in the fluorescence. The means of introducing a matching harmonic set of modulations in the fluorescent signal will be discussed below. Thus all the harmonics comprising the phase dependent signal at the output of the image intensifier device can be sampled for. This requires that the fundamental harmonic component be sampled at a rate sufficient to satisfy the Nyquist criterion for the highest harmonic component present. Ultimately the amount of sampling will be limited by the bandwidth of the detector. From a Fourier analysis of the single set of phase dependent images the pixel specific phase shift and demodulation necessary for spatially resolving heterogeneous lifetime can be calculated.

Before a detailed description of the apparatus of the present invention, the underlying theory will first be discussed.

Any excitation field (e.g. the excitation energy 2 of Figure 1a) repetitively modulated at frequency *f* can be represented as a Fourier series:

$$E(t) = E_0 + \sum_{n=1}^{\infty} E_n Cos(n\omega t + \Theta_n) \qquad (1)$$

where $\omega = 2\pi f$, and is the fundamental circular frequency of the modulation, E_0 is the time independent average intensity, and E_n is the intensity amplitude of the n^{th} harmonic frequency component with phase Θ_n.

After directing the excitation energy 2 at the sample 1, the resulting fluorescence emission 5 (=F(t)) contains the same harmonic content:

$$F(t) = QE\left(F_0 + \sum_{n=1}^{\infty} F_n Cos\left(n\omega t + \Theta_n^{'}\right)\right) \qquad (2)$$

QE is a multiplication factor accounting for photon detection efficiency, intensity of excitation and fluorophore quantum yield. Here, however, each harmonic term of the fluorescence measured relative to the equivalent term in the excitation field, has both a phase shift $\Delta\phi_n = \Theta_n - \Theta_n^{'}$ and demodulation $M_n = M_F / M_E = F_n E_0 / E_n F_0$ which vary to an extent dependent upon the fluorescence lifetime composition of the sample 1, given by the dispersion relationships:

$$\Delta\phi_n = Tan^{-1}\left(\sum_{q=1}^{Q} \frac{\alpha_q n\omega\tau_q}{1+\left(n\omega\tau_q\right)^2} \Big/ \sum_{q=1}^{Q} \frac{\alpha_q}{1+\left(n\omega\tau_q\right)^2}\right) \qquad (3)$$

$$M_n = \left(\left(\sum_{q=1}^{Q} \frac{\alpha_q n\omega\tau_q}{1+\left(n\omega\tau_q\right)^2}\right)^2 + \left(\sum_{q=1}^{Q} \frac{\alpha_q}{1+\left(n\omega\tau_q\right)^2}\right)^2\right)^{\frac{1}{2}} \qquad (4)$$

where $\alpha_q = a_q\tau_q$ is the fractional contribution to the steady state fluorescence from the q^{th} emitting species. The dispersion relationships given by equations 3 and 4 can be fitted at multiple frequencies to resolve lifetimes and corresponding amplitudes of samples containing composite fluorescent species (Gratton and Limkeman (1983) *Biophys. J.* **44**, 315; Lakowicz and Maliwal (1985) *Biophys. Chem.* **21**, 61).

From these parameters the average phase and modulation dependent fluorescence lifetimes can also be evaluated for each of the frequency components using the relationships:

$$< \tau_n^{phase} >= \frac{Tan(\Delta\phi_n)}{n\omega} \tag{5}$$

$$< \tau_n^{M} >= \frac{\sqrt{\frac{1}{M_n^{\,2}} - 1}}{n\omega} \tag{6}$$

A repetitive high frequency voltage modulation across either the MCP 7 or photocathode 6 provided by a frequency synthesiser results in a repetitive modulation of the gain characteristics G(t) at every pixel of the detector. Expanding this into a Fourier series gives:

$$G(t,k) = G_0 + \sum_{m=1}^{\infty} G_m \cdot Cos\left(m\omega t + \varphi_m + mk\Delta\varphi\right) \tag{7}$$

where G_0 is the average gain amplitude, G_m is the gain amplitude for every frequency harmonic with associated phase φ_m and $k\Delta\varphi$ is the adjustable phase setting of the frequency synthesiser, which may be sequentially incremented by $\Delta\varphi$. The MCP 7 response is proportional to the incident fluorescence intensity multiplied by the gain characteristics of the intensifier.

Where both these are given by general equations 2 and 7, the frequency mixing results in a signal 8 composed of a time invariant response, oscillations at the harmonics of the gain and fluorescence modulations, and a combination of oscillations at the sum and difference frequencies of the harmonics. The slow response time of the phosphor screen 9 at the output of the imaging device gives an integrated signal image $D(t,k)$ consisting only of the low frequency components of the total MCP response:

$$D(t,k) = QE\left(\frac{G_0F_0 + \frac{1}{2}\sum_{m=n=1}^{\infty} G_nF_nCos\left(n\Delta\omega t + \Theta_n' - \varphi_n' - mk\Delta\varphi\right)}{} \right) \quad (8)$$

where $\Delta\omega = |\omega - \omega'|$.

For the homodyne detection mode the mixing frequencies are chosen to give $\Delta\omega = 0$, resulting in a phase sensitive output image given by:

$$D(k) = QE\left(G_0F_0 + \frac{1}{2}\sum_{n=1}^{\infty} G_nF_n \cdot Cos\left(\phi_n - nk\Delta\varphi\right) \right) \quad (9)$$

Thus from equation (9), a series of images recorded sequentially at discrete phases $k\Delta\varphi$ can be used to map the time dependent fluorescence signal given by equation 2.

Fourier Decomposition and Analysis of mfFLIM phase dependent images.

Linearisation of equation (9) by standard trigonometric identities gives the constant (dc), cosine (a_n) and sine (b_n) components of the detected signal, where

$$D(k) = dc + \sum_{n=1}^{N} [a_n Cos\ (nk\Delta\varphi) - b_n Sin\ (nk\Delta\varphi)] \quad (10a)$$

$$a_n = ac_n Cos(\phi_n) \quad (10b)$$

$$b_n = ac_n Sin(\phi_n) \quad (10c)$$

By direct comparison of equations (9) and (10) it is seen that dc = $QE.G_0F_0$ gives the amplitude of the phase invariant signal and ac_n = $QE.G_nF_n/2$ gives the amplitudes of the harmonics. A phase dependent image is acquired after every phase step $\Delta\varphi$ in the gain repeated over a

single cycle (0–360°) of the fundamental frequency component of the signal. The total number of images is chosen to satisfy the Nyquist sampling criterion for the highest significant harmonic component in the phase dependent data i.e. $k >> 2N$. The cosine (a_n), sine (b_n) and dc terms in the Fourier expansion given by equation 10 may be obtained on a pixel by pixel basis from the set of phase dependent images, either by the application of a discrete Fourier transform (DFT) or by fitting to a Fourier expansion 'model' i.e. a band-limited form of equation 10, using a singular value decomposition (SVD) algorithm. For each harmonic the phase shift $\Delta\phi_n$ and demodulation M_n are calculated from the Fourier amplitudes (dc, a_n, b_n) of the fluorescence signal relative to a zero lifetime sample (ie. a scattering or reflective sample) according to:

$$\Delta\phi_n = \tan^{-1}\left(\frac{b_n}{a_n}\right) - \Psi_{E,n} \qquad (11)$$

$$M_n = \frac{ac_n}{dcM_{E,n}} = \frac{\sqrt{a_n^2 + b_n^2}}{dc \cdot M_{E,n}} \qquad (12)$$

Where $\Psi_{E,n}$ and $M_{E,n}$ are the phase and relative modulation of a reflected or scattered excitation light at each harmonic.

In the above analysis it has been implicitly assumed that both the fluorescent signal and gain characteristics of the detector have amplitude in the high harmonic content. This can be achieved in practice by a number of methods.

Excitation source

Excitation of a sample under analysis by a beam of excitation energy intensity modulated with a plurality of frequencies in a harmonic series can be achieved in several ways.

In a first embodiment, a pulsed laser source is used. For instance mode-locked lasers typically have pulse repetition rates of approximately 80 MHz. This is ideal for measuring nanosecond fluorescence lifetimes. The pulse width from such a laser is typically less than 50 picoseconds which is sufficiently short in comparison to the period ($1/f$) to produce a broad excitation spectrum which will include significant amplitude in many of the higher harmonic terms ie. 160, 240, 320.... MHz. If a lower fundamental frequency is required, a mode locked laser can be employed in combination with a pulse picker (typically performed using a Pockels Cell).

Alternatively, in a second embodiment, standing wave acousto-optic modulators (AOMs) provide a means of modulating a continuous wave laser in a sinusoidal manner at high frequencies (e.g. 10's-100's of MHz) to produce a much more limited excitation spectrum. Combinations of these AOMs can be employed in series to modulate the excitation light with a combination of frequencies corresponding to the modulation frequencies of all the individual AOMs, their difference frequencies and their sum frequencies.

Careful choice of AOM modulation frequency is preferable to ensure that the frequency content of the excitation corresponds to an harmonic series. This approach is used in a preferred embodiment of the present invention to be described later in connection with Figure 4.

With the technique of this second embodiment, the number of harmonic components making up the excitation energy spectrum, E(t) is highly constrained. It may then not be necessary to sample the output to satisfy the Nyquist criterion for the highest harmonic possible as determined by the detector bandwidth, merely only to the highest harmonic present in the excitation spectrum. Thus, problems associated with aliasing when sampling the phase dependent output can be reduced or eliminated. Furthermore, the reduction in the sampling requirements has significant benefits with respect to lifetime imaging of microscopic samples, where photobleaching and acquisition times impose significant experimental constraints.

Detector system

The polarity of any voltage applied to the photocathode of an image intensifier acts as a switch to the flow of photoelectrons, where a change in polarity, from negative to positive, switches off this flow. Thus, in a general sense, the application of a periodic zero-crossing control voltage results in a square wave type modulation of the gain characteristics of the device as the periodic voltage alternates in polarity. Preferably, a sinusoidal control voltage is used. By controlling the bias, or DC component, of the sinusoidal control voltage it is possible to control the duration of the 'on' state of the device relative to its 'off' state. In this way the width of the square pulse waveform can be used to control the amplitude of the higher harmonic content.

It is therefore possible to modulate the output of the detector with a modulation function having frequency components corresponding to some or all of the harmonic series of the excitation energy irradiating the sample under analysis. Preferably, the sinusoidal control voltage has a frequency

which corresponds to the lowest (fundamental) frequency of the harmonic series of the excitation energy modulation.

Modulation depths of up to 400% are possible from this form of modulation. Frequency synthesisers are widely available for providing a highly stable, high frequency sinusoidal voltage source (kHz–GHz) on top of a controllable bias voltage.

The determination of photoelectron gain as a function of photocathode bias for an exemplary image intensifier unit (the Hamamatsu C5825) is shown in Figure 2. An experimental configuration (corresponding to the image intensifier configuration in Figure 1) for the measurement is shown in Figure 2a, comprising elements photocathode 6, microchannel plate 7, phosphor screen 9 and CCD camera 10. The Hamamatsu C5825 bias voltage is controllable via a ten-turn potentiometer on its power supply module from –50V to +25V, with a resolution of approximately ± 0.01V.

At positive voltages the device is in an 'off' state, and a rapid switch to the 'on' state occurs for a small negative bias as illustrated in Figure 2b, which shows the response of the image intensifier device to constant illumination. For increasingly negative voltages, after the initial step 20, the gain characteristics of the intensifier increase linearly in the portion 21 of the transfer characteristic 22.

From the photoelectron gain function thus plotted, the response of the image intensifier to the application of a sinusoidal photocathode bias voltage 23 as shown in Figure 2c can be shown as that in Figure 2d. This response is obtained from the graphical projection of the sinusoidal photocathode voltage onto the photoelectron transfer plot. To a good

approximation, this represents a square pulse wave type response 24, and throughout the present specification, this will be generally referred to as a square pulse wave. The width 25 of the square pulse wave modulation can be controlled by changing the photocathode bias 26 (Figure 2a).

A square pulse wave modulation, of arbitrary width D, is given in Figure 2e. G(t) is periodic in time ie. G(t) = G(t + T) and G(t) = K for $0<t<D$.

Expanding as a Fourier series:

$$G(t)= G_0 +\sum_{n=1}^{N} G_n Cos(n\omega t + \theta_n) \quad (10)$$

and rewriting using the standard trigonometric identity:

$$G(t) = G_0 +\sum_{n=1}^{N} a_n Cos(n\omega t) - b_n Sin(n\omega t) \quad (11)$$

where $a_n = G_n Cos(\theta_n)$ and $b_n = G_n Sin(\theta_n)$. (12)

The Fourier coefficients G_0, a_n and b_n can be evaluated using the following set of standard equations:

$$G_0 = \frac{1}{T}\int_{-T/2}^{T/2} G(t).dt = \frac{1}{T}\int_0^D K.dt \quad (13)$$

$$a_n = \frac{2}{T}\int_{-T/2}^{T/2} G(t)Cos(n\omega t).dt = \frac{2}{T}\int_0^D KCos(n\omega t).dt \quad (14)$$

$$b_n = \frac{2}{T}\int_{-T/2}^{T/2} G(t)Sin(n\omega t).dt = \frac{2}{T}\int_0^D KSin(n\omega t).dt \quad (15)$$

Solving the above gives:

$$G_0 = \frac{KD}{T} \quad (16)$$

$$a_n = \frac{K}{n\pi}.Sin\left(\frac{2n\pi D}{T}\right) \quad (17)$$

$$b_n = \frac{K}{n\pi}.\left[1 - Cos\left(\frac{2n\pi D}{T}\right)\right] \qquad (18)$$

From the sine and cosine components the modulation and phase can be determined :

$$G_n = \sqrt{a_n^2 + b_n^2} \quad \text{and} \quad \theta_n = Tan^{-1}\left(\frac{b_n}{a_n}\right) \qquad (19)$$

From the above the relative modulation depth is:

$$M_G = \frac{G_n}{G_0} = \frac{2}{nr\pi}\sqrt{2 - 2Cos(2nr\pi)} \qquad (20)$$

where $r = D/T$ and is the fractional square pulse width.

Figure 3 shows the variation in the modulation depth against r for the first five harmonic terms of the gain, where G_0 is the fundamental, and $G_1 \ldots G_5$ are the first five harmonics.

Relative modulation depths up to 400% are possible for sufficiently narrow pulse widths. This is four times greater than the maximal modulation depth possible with pure sine wave modulation. The higher modulation comes at a cost to detection sensitivity.

With existing technology, detectors can operate satisfactorily with modulated outputs as high as 360 MHz.

With reference now to Figure 4, a presently preferred embodiment of a multiple frequency fluorescence lifetime imaging apparatus 40 will now be described.

Instrument Configuration

The continuous wave output 42 of an Argon/Krypton laser 41 is passed through a pair of AOMs 43, 44 (either a 40 MHz or 160 MHz in combination with an 80 MHz standing wave AOM 44). The output light source excitation beam 45 is thereby sinusoidally modulated at high frequencies at both the fundamental AOM frequencies, and the sum and difference frequencies, to provide a harmonic set of modulation frequencies from 20 MHz to 340 MHz, which are used to excite sample 1 within microscope 71.

The AOMs 43, 44 are respectively driven by the amplified voltages 50, 51 from two (slave) frequency synthesisers 52, 53 which are phase locked to a third (master) frequency synthesiser 60 using a first output 60a. The third frequency synthesiser 60 also has a second, phase-adjustable, output 60b. This is used to derive, via amplifier 61, a gain control signal 62 used to modulate the photocathode voltage of an image intensifier 70, at the fundamental harmonic frequency of the excitation beam 45.

(It will be understood that the photocathode voltage could alternatively be modulated at one of the harmonic frequencies of the excitation beam, but that this would reduce the number of higher harmonics in the gain response $G(t)$ for which lifetimes can be calculated, and would thus be less efficient.)

The relative phase difference between the excitation beam 45 and the gain control signal 62 can be controlled by the phase-adjustable output 60b, enabling phase dependent image collection. This is preferably carried out under the control of a computer system 81 by control bus 82.

Iris diaphragms 46, 47 placed approximately 1.5 metres from each of the AOMs 43, 44 select the zero from the higher order diffracted beams (6.4 mrad beam separation) and a variable neutral density wheel 48 of 0–5 OD provides for control of the overall signal intensity.

To eliminate the effect of laser speckle at the sample 1, the high spatial and temporal coherence properties of the laser beam are removed by passing the light through a rotating ground glass disc 49.

The scattered radiation from the rotating ground glass disc 49 is collected and collimated with a high numerical aperture lens 58 before being directed into the epi-illumination port of an inverted microscope 71 which results in Köhler illumination at the sample 1.

Steady state fluorescence images are captured using a scientific grade CCD camera 10. For recording lifetimes, the sample fluorescence is first imaged onto the photocathode 9 of a C5825 image intensifier (Figure 2a). A telescopic lens 72 optically couples the phase dependent image at the phosphor screen 9 output to the CCD camera 10 and images are downloaded over an AIA bus 80 to an interface card in the computer system 81.

Preferably, the computer system 81 also provides positional control and feedback of a motorised stage 83 on which the sample is mounted, by way of a control unit 84 and interface bus 85.

A series of phase dependent images are taken over the full cycle (0–360°) of the fundamental harmonic, where the number of images satisfies

Nyquist's criterion for the full harmonic content present in the homodyne signal.

The resulting sampled output is then passed to the computer system 81 where a Fourier analysis can be performed.

Figure 5 shows exemplary measurements of relative harmonic content present in the gain characteristics of the C5825 image intensifier as function of photocathode relative bias voltage. The relative bias voltage is defined as the photocathode offset voltage divided by the amplitude of the applied sinusoidal voltage. These results were obtained by measuring a cycle of phase dependent images from a reflecting sample 1 illuminated with pure sinusoidally modulated laser light which is an integer multiple of the driving frequency of the image intensifier. The relative amplitude at each harmonic is obtained by Fourier analysis. A photocathode bias signal comprising a ±17.8 V sinusoid plus a variable bias is applied to the photocathode 6. The average gain in Figure 5a has been normalised to its maximum value and in Figures 5 (b-e), the relative modulation depths in the gain are scaled by half the relative modulation depth of the reflected excitation. The data for each figure was acquired with the fundamental frequency of the reflected light and photocathode voltage respectively set to (a, b) 80.236 and 80.236 MHz, (c) 80.236 MHz and 40.118 MHz, (d)80.238 MHz and 26.746 MHz and (e)80.236 MHz and 20.059 MHz.

Typical examples of phase sampled excitation light from a reflecting sample and its relative harmonic are shown in Figure 6. Because the sample (a piece of aluminium foil) has no lifetime associated with it these figures essentially show the instrumental response of the detector to the excitation modulations. The data shown in Figure 6a, designated as the

"low" frequencies set,was obtained with the 40MHz and 80MHz AOMs 43, 44 set to modulate the excitation light at frequencies of 42.154 MHz and 63.231 MHz respectively; where frequency mixing gave rise to additional modulations with amplitudes greater than 0.1 at 21.077MHz, 105.385MHz and 126.462MHz. The data shown in Figure 6b, designated as a "high" frequency set, was obtained with the 80MHz and 160MHz AOMs 43, 44 set to modulate at a frequencies of 80.244 MHz and 160.488 MHz respectively, giving additional modulations in intensity with amplitudes greater than 0.1 at 240.732 MHz and 320.976 MHz. The relative bias on the photocathode of the image intensifier was set at about 0.9. The upper figures of Figure 6a and b plot the average intensity from the phase-sampled images of the reflective sample. The corresponding harmonic content in the data obtained, from a Fourier analysis, is shown in the lower figures respectively.

The mfFLIM configuration and fitting routines were tested on an equi-Molar (1 μM) solution of rhodamine 6G and rhodamine B in distilled water. The excitation light was modulated by the 40MHz and 80Mhz AOMs tuned 42.154 MHz and 63.231 MHz (including the sum and difference mixing frequencies). Homodyne detection of all the excitation frequencies was achieved by modulating the photocathode of the MCP at a fundamental frequency of 21.077 with a relative voltage bias of about 0.9, and 32 phase sampled images were collected in each case (i.e. 11.25^0 phase steps between images). Reference images were obtained by phase sampling reflected light from aluminium foil illuminated with the modulated laser light. All phase dependent images were corrected for dark current and stray light by subtracting an image acquired in the absence of excitation field illumination. No significant photobleaching was observed

in the fluorescence signal, due in main to the large diffusional volume of the sample.

Table 1 lists the results of fitting the phase shift and demodulation images generated from a Fourier analysis of the set of phase dependent images to the dispersion relationships (equations 3 and 4), based on a bi-exponential lifetime model. The lifetime values of 1.29 ns and 3.74 ns correspond well with listed literature values of 1.5ns and 4ns for rhodamine B and 6G respectively (Lakowicz & Berndt (1991). Rev. Sci. Instrum. **62**, 1727). A relative intensity contribution of 73% was recovered for the fluorescence from rhodamine 6G (α_1) which was in excellent agreement with a 71% value calculated from independent intensity measurements of rhodamine 6G and rhodamine B alone using the same filter set as for the mfFLIM experiment.

Table 1: Fluorescence lifetime fit parameters of rhodamine B/6G mixture.

	avg[1]	sdev[2]	<err>[3]
α_1	0.73	0.15	0.15
τ_1 (ns)	1.29	0.52	0.53
τ_2 (ns)	3.74	0.41	0.39

It will be noted that the number of frequencies in the harmonic series present in the excitation and gain modulation spectrum should be at least as many as the number of different lifetime species or states expected in the sample 1 in order to be able to resolve each lifetime species or state by fitting the data to the dispersion relationships.

In preferred modes of use, the excitation spectra of the detector systems of the present invention are calibrated by using a scattering surface sample 1 having no fluorescence lifetime characteristics. This enables determination of the optical and electrical path lengths of the detection system and the phase and modulation shifts caused thereby. This information is stored by the computer system 81 and used to compensate the output measurements taken.

Finally Figure 7 shows a practical application of the mfFLIM instrument. Here it is shown that mfFLIM can be used to disentangle the cellular distributions of two co-expressed green fluorescent protein mutants with differing lifetimes. In Hela cells a construct of the Golgi localisation signal N-acetylylglucosaminyltransferase I fused to the green fluorescent protein mutant GFP5 (ZernickaGoetz et al. (1997), Development **124**: 1133). (referred to as NA-GFP5) was co-expressed with the novel green fluorescent protein mutant YFP5 (which will distribute in the cytosol and nucleus). YFP5 is described in UK Patent co-pending application entitled "Fluorescent Protein" which has the same filing date as this application. Because of a high degree of spectral overlap it is difficult to efficiently isolate the fluorescence of these two GFP mutants by conventional filtering. In mfFLIM measurements a long pass dichroic and broadband emission filter can be used in order to simultaneously collect the fluorescence from both the GFP mutants. Figure 7a shows the dc component of the set of phase dependent images taken of one of the expressing Hela cells. Figures 7b and 7c show the isolated fluorescence from the GFP5 and YFP5 respectively, calculated by fitting the phase shift and demodulation images to the dispersion relationships based on a bi-exponential decay model.

Improving mfFLIM data analysis

Multiple parallel measurements of the time-resolved fluorescence kinetics of the probes in different states are obtained in mfFLIM ie. each pixel in the image can be conceived of as an individual experiment. Global analysis of a multiple of such experiments has a clear advantage over individual analysis of the data at a single point (Beechem (1992) Methods Enzymol. **210**, 37). Inter-relationships between decay parameters at each pixel can be encoded in a global fit of the image in order to significantly reduce the fitting errors. For example, for a mixture of two fluorophores with homogeneous decays or a single fluorophore in two states, the lifetimes are pixel invariant and could be linked in the double exponential decay fitting model over the whole image where the amplitudes are left uncoupled. This results in a reduction of $2N^2$-2 fitting parameters for an N×N image Furthermore by using a global fitting approach with mfFLIM data it should be possible to significantly reduce the number of frequency measurements necessary to achieve estimates of fluorescent lifetime parameters with a specified signal to noise ratio. This is critical with microscopic samples where the data acquisition time and total exposure have to be carefully controlled. The measurements of the populations of GFP fusion proteins or fluorescence resonance energy transfer (FRET) through donor and acceptor tagged proteins in cells would be examples of systems where the global fitting approach might be expected to bring significant improvements in quantifying the populations or states of molecules.

CLAIMS

1. A method for making fluorescence lifetime measurements comprising the steps of:

irradiating a fluorescent material with a beam of excitation energy intensity modulated with a plurality of frequencies in a harmonic series including a fundamental frequency and at least one harmonic thereof;

receiving a fluorescence emission from said fluorescent material into a detector having a controllable gain and an output;

modulating the output of said detector using said controllable gain, with a modulation function having frequency components corresponding to at least two of said harmonic series of said irradiating beam, and having a controllable phase angle relationship thereto;

sampling said detector output to determine an amplitude for each of a plurality of phase angles.

2. A method according to claim 1 further including the step of:

selecting the number of frequency components in said harmonic series to be at least as many as the number of different lifetime components to be measured in the fluorescent material.

3. A method according to claim 1 or claim 2 in which the step of irradiating the fluorescent material includes excitation with a pulsed laser source to generate a broad modulation spectrum inclusive of components at each frequency of said harmonic series.

4. A method according to claim 1 or claim 2 in which the step of irradiating the fluorescent material includes the step of modulating a continuous wave laser source using a plurality of acousto-optic modulators

selected to generate said harmonic series with their individual, sum and difference frequencies.

5. A method according to any preceding claim wherein the detector includes an image intensifier device and in which the modulating step comprises the step of applying a periodic bias voltage to the photocathode of the image intensifier so as to result in square wave modulation of the gain characteristics of the image intensifier device.

6. A method according to claim 5 in which the periodic bias voltage comprises a sinusoidal voltage having a peak-to-peak voltage and a DC component sufficient to cause negative-going excursions for only a small portion of its negative half-cycle.

7. A method according to any preceding claim in which the sampling step includes the step of sampling the detector output over a range of phase angles relative to the lowest frequency component of the modulation signal, the number of sampled phase angles being sufficient to resolve the highest frequency component of the harmonic series in the detector output.

8. A method according to claim 7 in which the number of components in the excitation energy is substantially restricted to only said harmonic series so as to reduce sampling requirements.

9. A method according to claim 7 in which the number of sampled phase angles is sufficient to resolve the highest frequency component of the detector.

10. A method according to claim 1 further including the step of using said sampled output to determine the phase and modulation lifetimes of said fluorescent material at each of said modulation signal frequencies, by:

carrying out a Fourier transform on said sampled output of the detector to determine the amplitude of the DC and AC components of the harmonic series;

for each frequency of the harmonic series, determining the phase shift and demodulation; and

for each frequency of the harmonic series, evaluating the phase and modulation lifetimes.

11. A method according to claim 1 further including the step of determining the lifetime composition of the sample by the steps of:

carrying out a Fourier transform on said sampled output of the detector to determine the amplitude of the DC and AC components of the harmonic series;

for each frequency of the harmonic series, determining the phase shift and demodulation; and

fitting the phase shifts and demodulations to the dispersion relationships of equations (3) and (4).

12. A method according to claim 10 further including:

simultaneously carrying out the steps of claim 10 for each of a plurality of spatial locations in the fluorescent material; and

simultaneously determining the spatial variation in phase and modulation lifetimes of said fluorescent material.

13. A method according to claim 12 in which the fluorescent material comprises a mixture of at least two components each having a

predetermined interrelationship between decay parameters, in which said step of simultaneously determining the spatial variation in phase and modulation lifetimes includes using a global fitting algorithm incorporating said predetermined interrelationship to reduce fitting errors.

14. A method according to claim 13 in which said predetermined interrelationship includes said fluorescent material components each having a lifetime which is invariant across said plurality of spatial locations.

15. A method according to claim 12 in which the detector includes an image intensifier including a microchannel plate device, the method further including the steps of:

imaging the fluorescent material onto the photocathode of an image intensifier;

simultaneously amplifying the photocathode output for each of a plurality of pixels of the image using the microchannel plate device;

determining the amplitude of the output for each of the plurality of phase angles for each pixel of the image; and

using said sampled output to determine the phase and modulation lifetimes of said fluorescent material at each of said modulation signal frequencies for each pixel of the image.

16. A method of imaging spatial variations in fluorescence lifetimes in a sample comprising the steps of:

irradiating a fluorescent material with a beam of excitation energy intensity modulated with a plurality of frequencies in a harmonic series including a fundamental frequency and at least one harmonic thereof;

imaging a fluorescence emission from said fluorescent material onto a detector having a controllable gain and an output;

modulating the output of said detector using said controllable gain, with a modulation function having frequency components corresponding to at least two of said harmonic series of said irradiating beam, and having a controllable phase angle relationship thereto; and

generating an image of spatial variation in the modulated detector output for each of a plurality of phase angles between said excitation beam and said modulation signal.

17. Apparatus for making multi-frequency fluorescence lifetime measurements comprising:

a source of electromagnetic radiation for irradiating a fluorescent material with a beam of excitation energy intensity modulated with a plurality of frequencies in a harmonic series including a fundamental frequency and at least one harmonic thereof;

means for receiving a fluorescence emission from said fluorescent material into a detector having a controllable gain and an output;

means for modulating the output of said detector using said controllable gain, with a modulation function having frequency components corresponding to at least two of said harmonic series of said irradiating beam, and having a controllable phase angle relationship thereto;

means for sampling said detector output to determine an amplitude for each of a plurality of phase angles.

18. Apparatus for imaging spatial variations in fluorescence lifetimes in a sample, comprising:

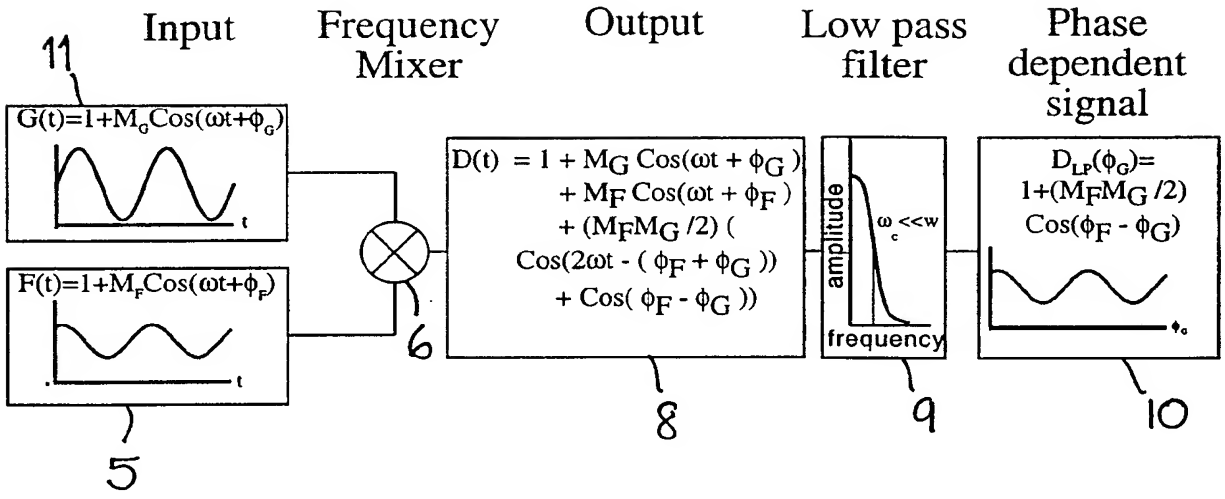
a source of electromagnetic radiation for irradiating a fluorescent material with a beam of excitation energy intensity modulated with a plurality of frequencies in a harmonic series including a fundamental frequency and at least one harmonic thereof;

means for imaging a fluorescence emission from said fluorescent material onto a detector having a controllable gain and an output;

means for modulating the output of said detector using said controllable gain, with a modulation function having frequency components corresponding to at least two of said harmonic series of said irradiating beam, and having a controllable phase angle relationship thereto; and

means for generating an image of spatial variation in the modulated detector output for each of a plurality of phase angles between said excitation beam and said modulation signal.

Fig. 1b


Input
Frequency Mixer
Output
Low pass filter
Phase dependent signal
11
G(t)=1+M_G Cos(ωt+φ_G)
F(t)=1+M_F Cos(ωt+φ_F)
5
6
D(t) = 1 + M_G Cos(ωt + φ_G) + M_F Cos(ωt + φ_F) + (M_F M_G /2) (Cos(2ωt - (φ_F + φ_G)) + Cos(φ_F - φ_G))
8
amplitude
frequency
ω_c <<w
9
D_LP(φ_G)= 1+(M_F M_G /2) Cos(φ_F - φ_G)
10

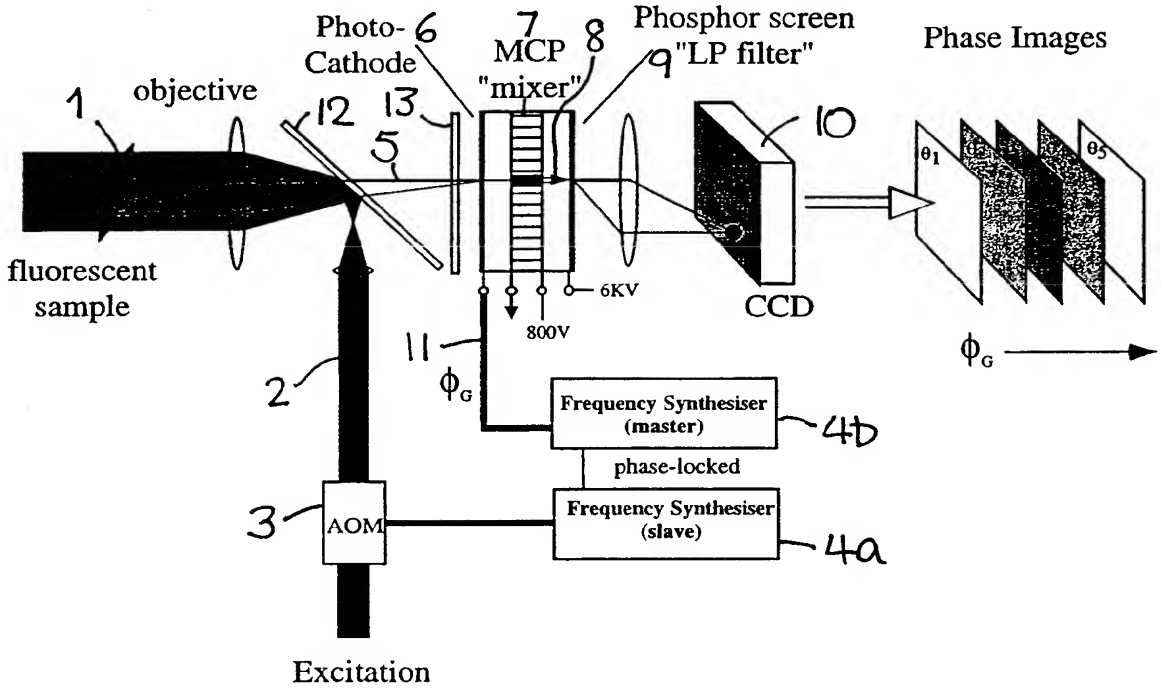


Photo-Cathode
6
7
MCP "mixer"
8
Phosphor screen
9 "LP filter"
Phase Images
1
objective
12
13
5
10
fluorescent sample
6KV
800V
CCD
θ1
θ5
φ_G
11
φ_G
2
Frequency Synthesiser (master)
4b
phase-locked
3
AOM
Frequency Synthesiser (slave)
4a
Excitation

Fig. 1a

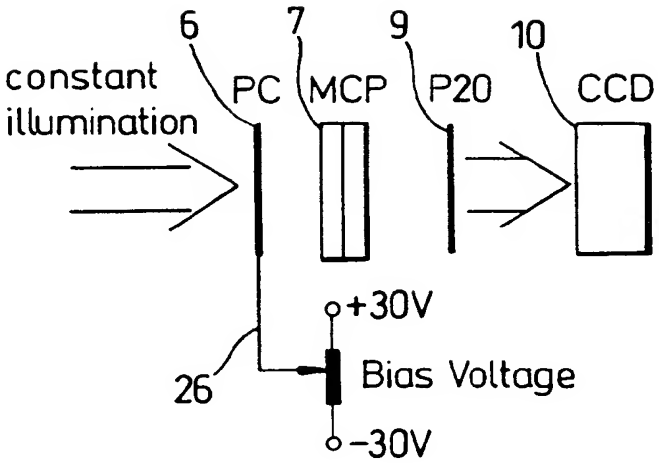

6
7
9
10
constant
illumination
PC
MCP
P20
CCD
+30V
Bias Voltage
26
-30V

Fig. 2a

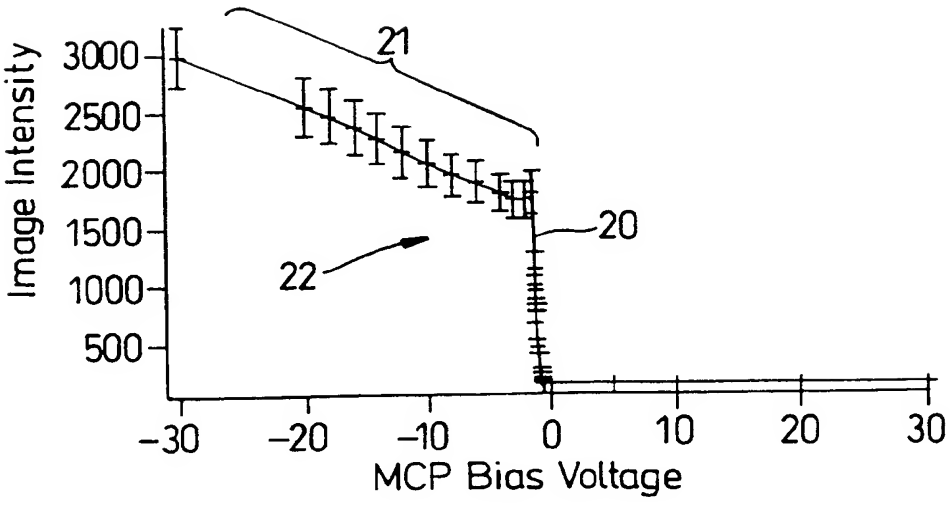


Image Intensity
3000
2500
2000
1500
1000
500
21
20
22
-30
-20
-10
0
10
20
30
MCP Bias Voltage

Fig. 2b

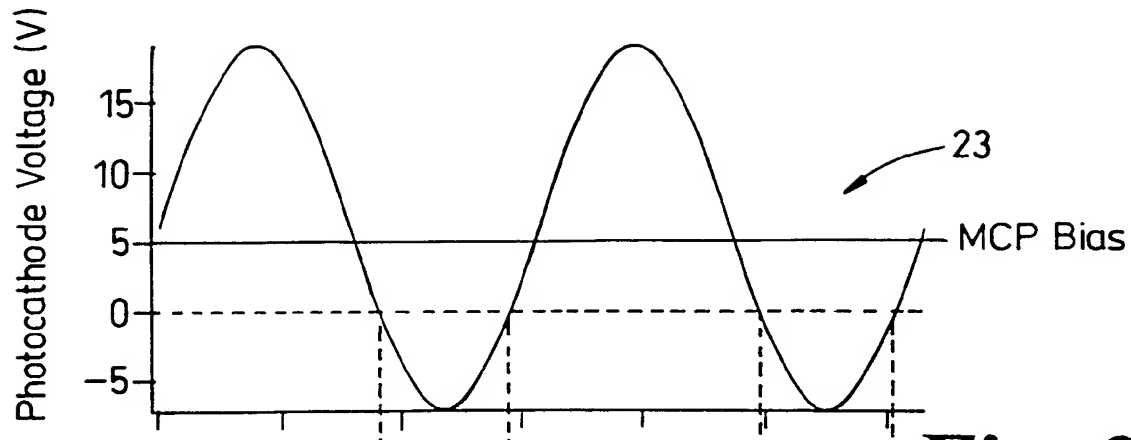


Fig. 2c

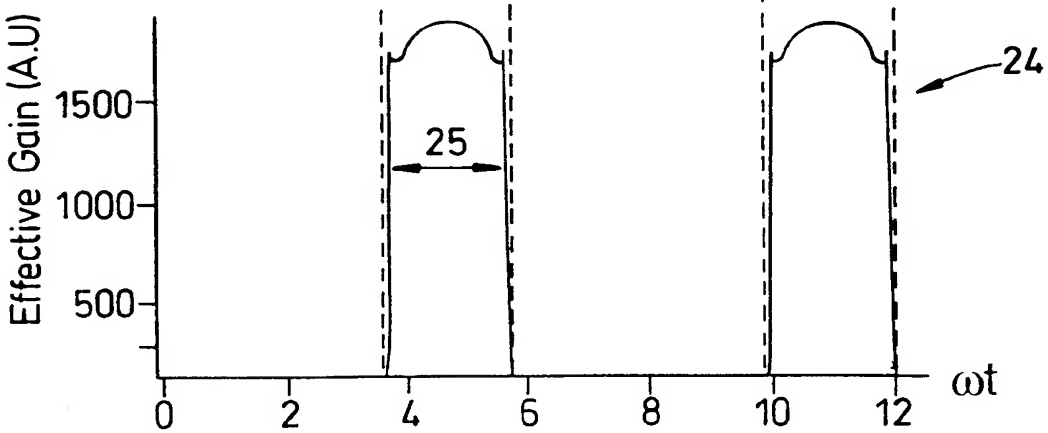


Fig. 2d

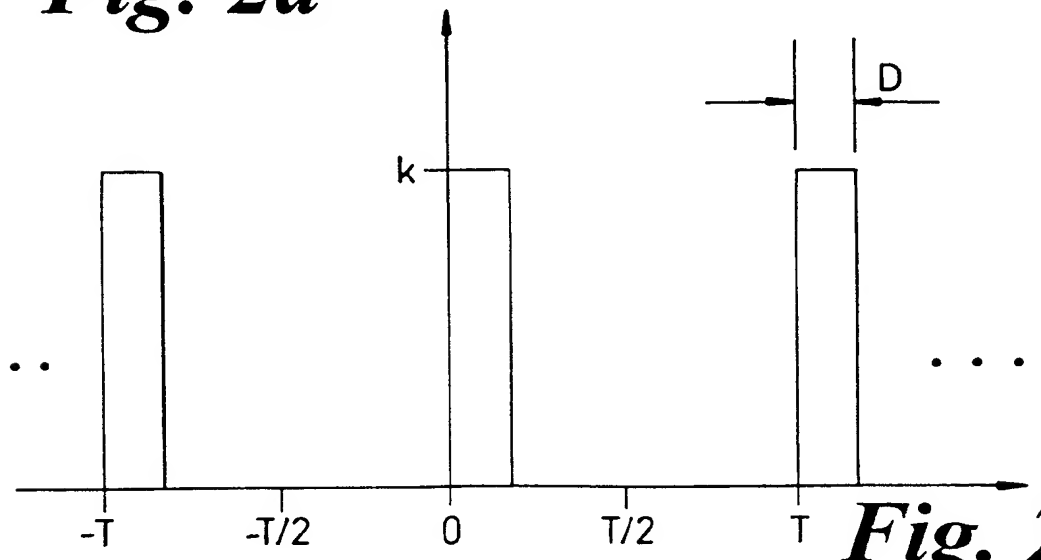


Fig. 2e

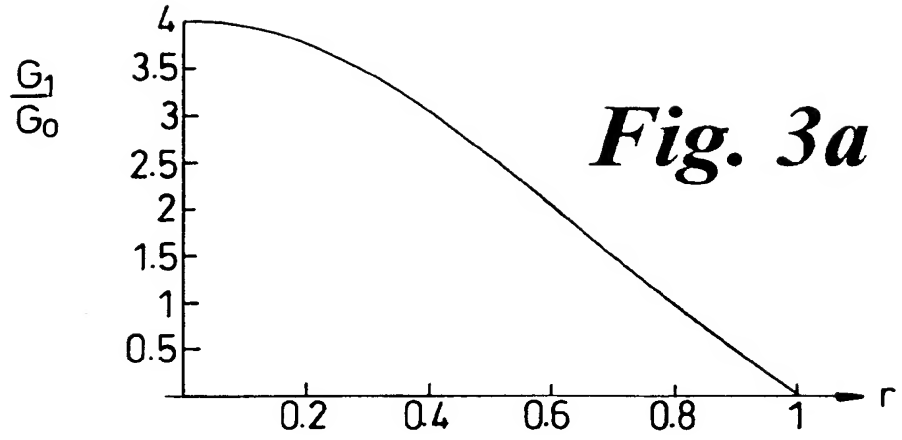


Fig. 3a

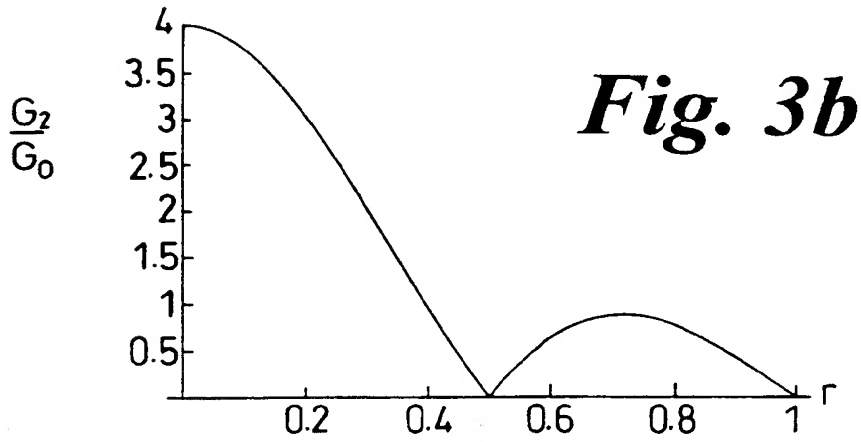


Fig. 3b

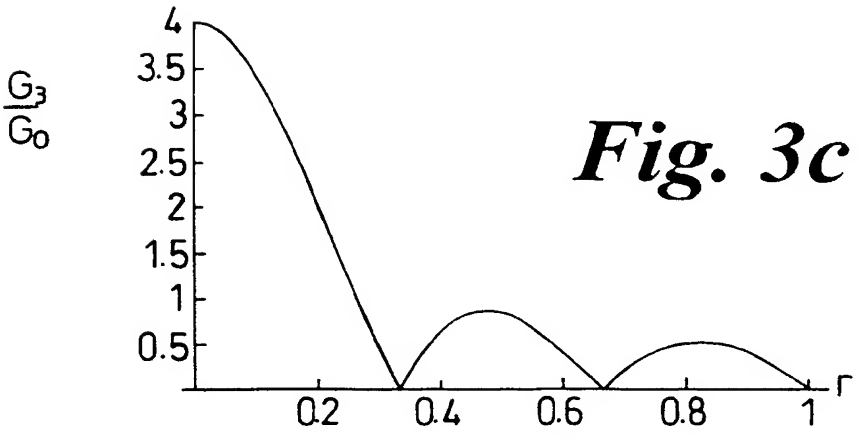


Fig. 3c

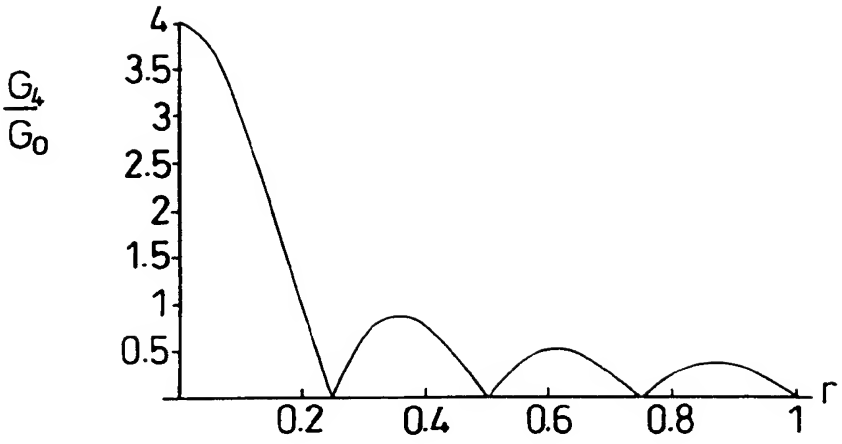

G4/G0
4
3.5
3
2.5
2
1.5
1
0.5
0.2
0.4
0.6
0.8
1
r

Fig. 3d

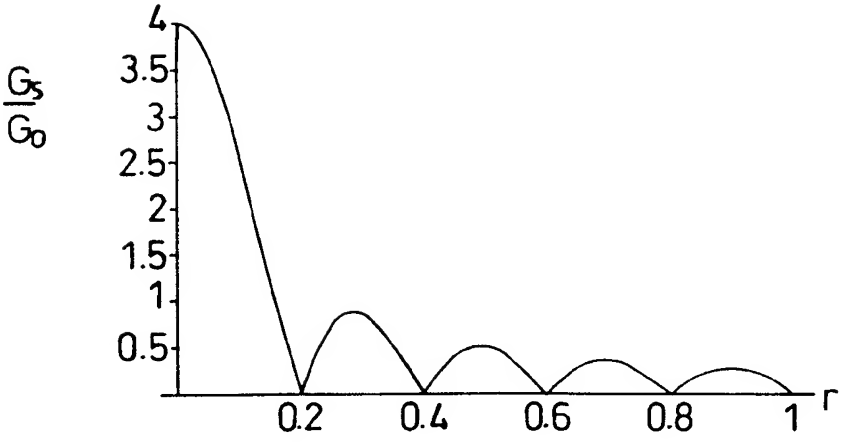

G5/G0
4
3.5
3
2.5
2
1.5
1
0.5
0.2
0.4
0.6
0.8
1
r

Fig. 3e

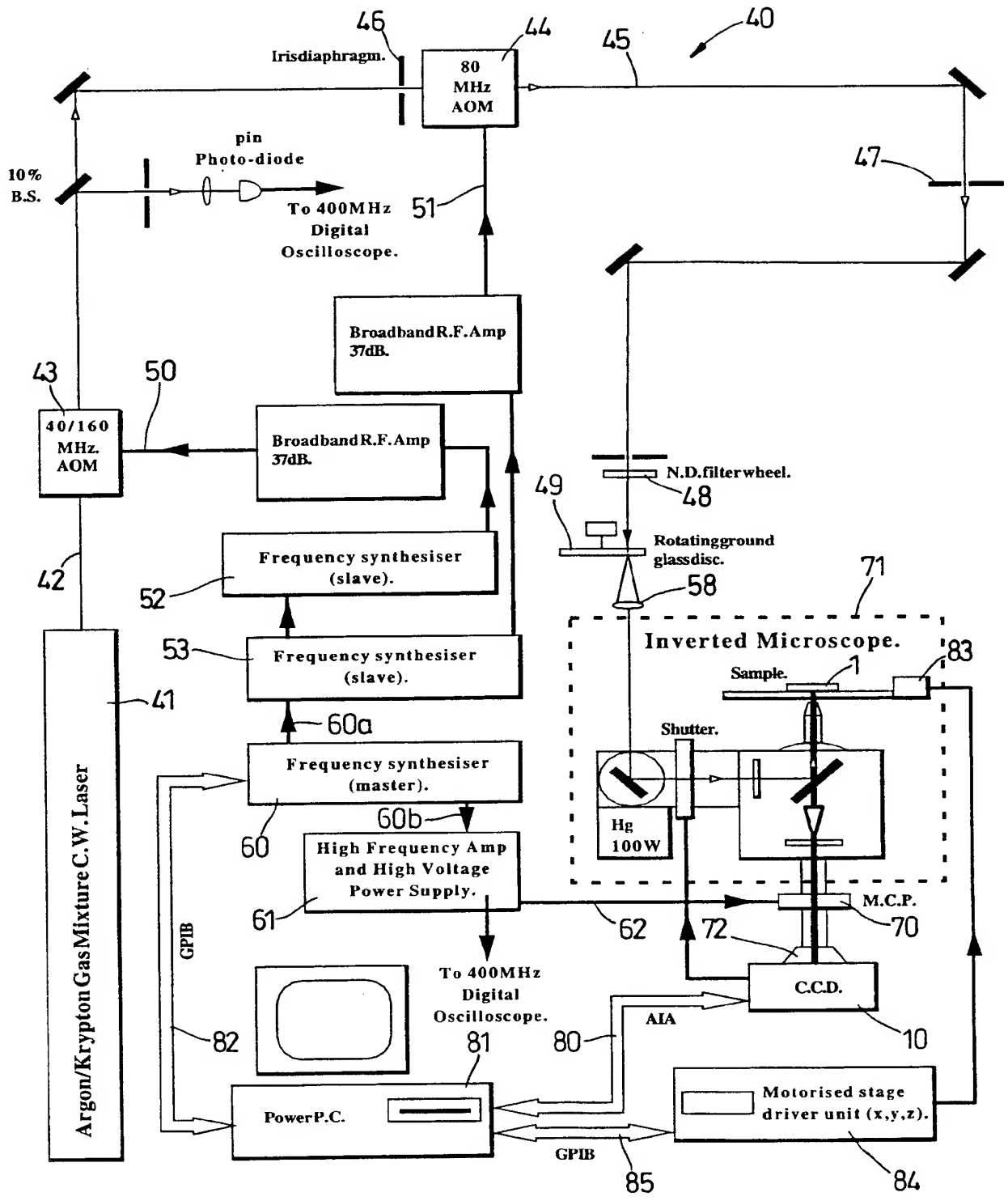

46
Irisdiaphragm.
44
80 MHz AOM
45
40
pin Photo-diode
10% B.S.
To 400MHz Digital Oscilloscope.
51
47
BroadbandR.F.Amp 37dB.
43
50
40/160 MHz. AOM
BroadbandR.F.Amp 37dB.
N.D.filterwheel.
49
48
Rotatingground glassdisc.
Frequency synthesiser (slave).
42
52
58
71
Inverted Microscope.
53
Frequency synthesiser (slave).
Sample.
1
83
41
60a
Shutter.
Frequency synthesiser (master).
Hg 100W
60b
60
High Frequency Amp and High Voltage Power Supply.
M.C.P.
61
62
72
70
GPIB
To 400MHz Digital Oscilloscope.
C.C.D.
AIA
82
81
80
10
Argon/Krypton Gas Mixture C.W. Laser
PowerP.C.
Motorised stage driver unit (x,y,z).
GPIB
85
84

Fig. 4

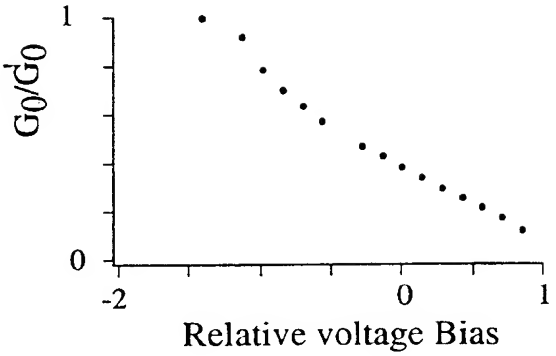


Fig. 5a

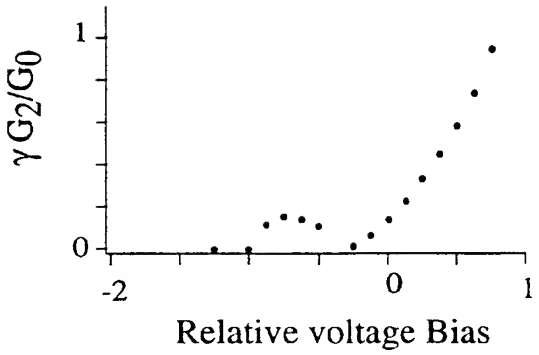


Fig. 5d

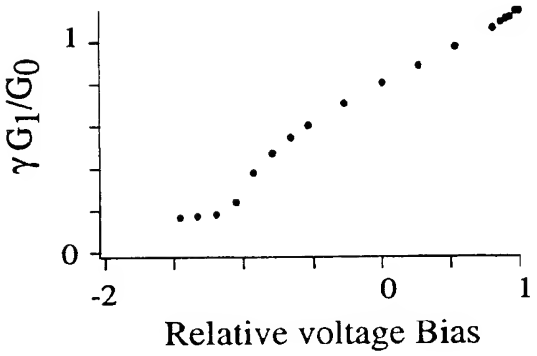


Fig. 5b

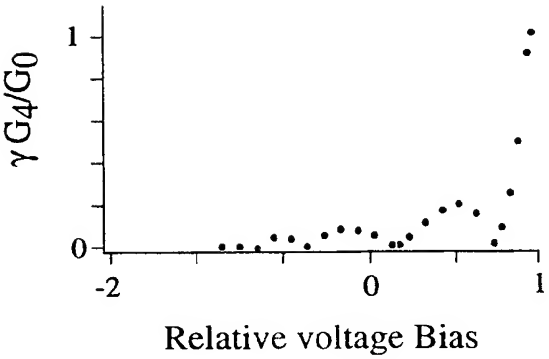


Fig. 5e

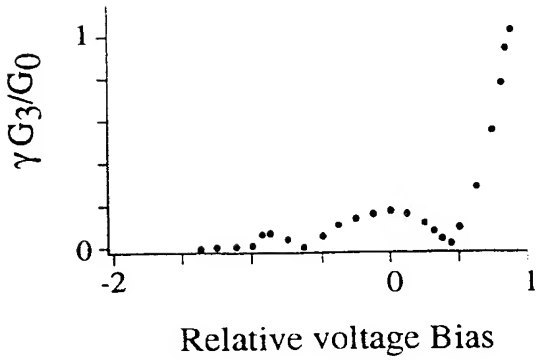
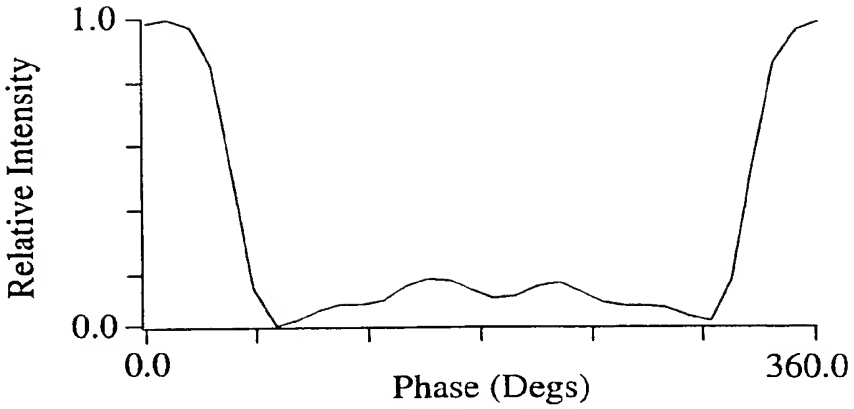


Fig. 5c


1.0
Relative Intensity
0.0
0.0
Phase (Degs)
360.0

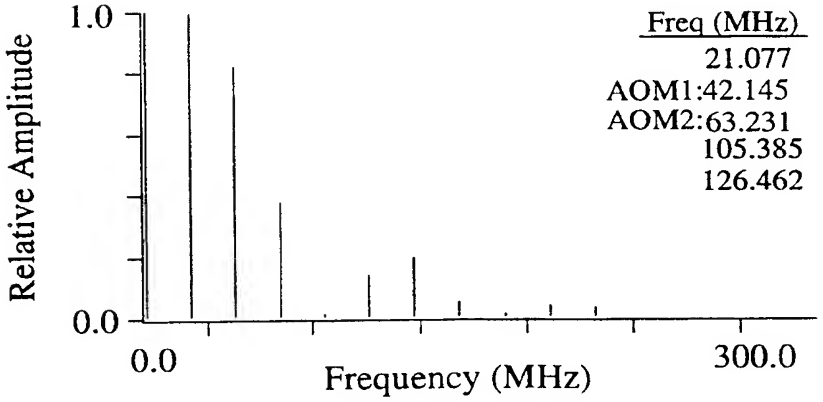
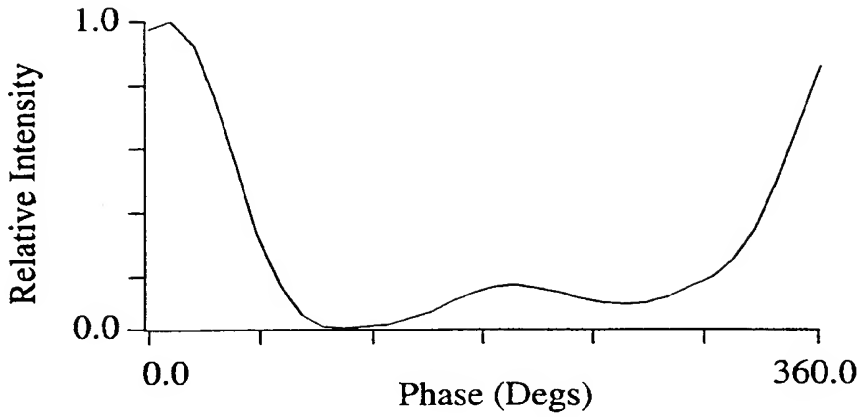

1.0
Relative Amplitude
0.0
0.0
Frequency (MHz)
300.0
Freq (MHz)
21.077
AOM1:42.145
AOM2:63.231
105.385
126.462

Fig. 6a


1.0
Relative Intensity
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Phase (Degs)
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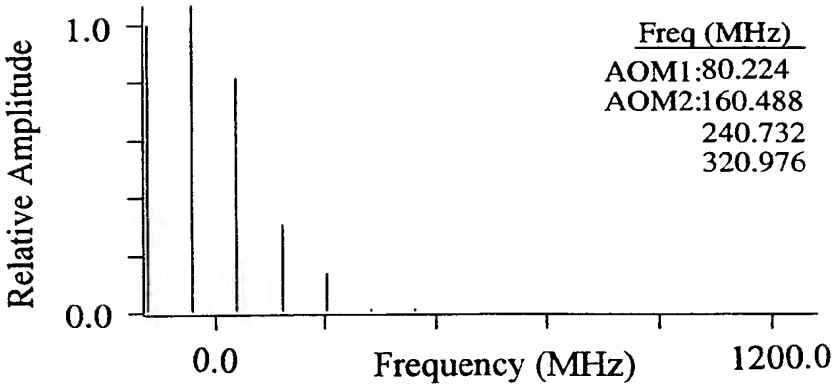
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Relative Amplitude
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0.0
Frequency (MHz)
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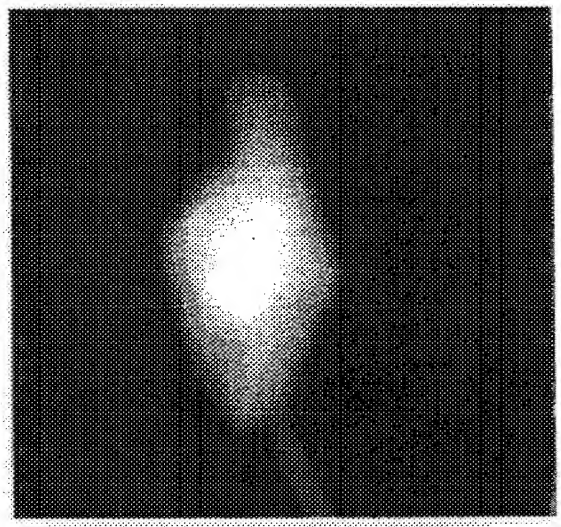
Freq (MHz)
AOM1:80.224
AOM2:160.488
240.732
320.976

Fig. 6b

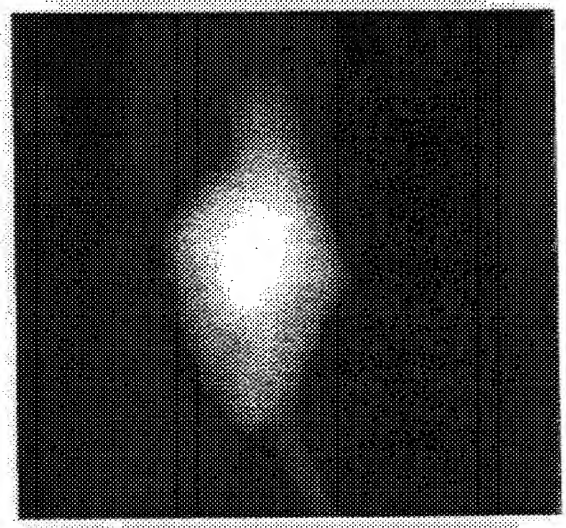
dc

Fig. 7a



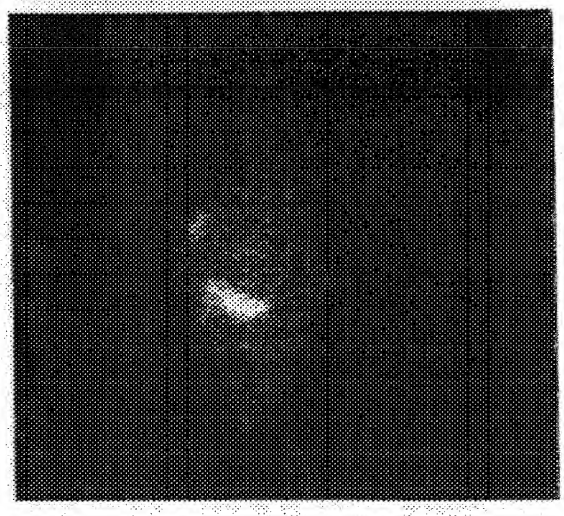
dc-YFP5

Fig. 7b



dc-NAGFP5

Fig. 7c



INTERNATIONAL SEARCH REPORT

Internat ' Application No
PCT/GB 99/02597

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N21/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 90 09637 A (RESEARCH CORPORATION TECHNOLOGIES) 23 August 1990 (1990-08-23) page 8, last paragraph page 13, line 6 - line 12 page 15, line 1 -page 16, line 6 page 17, line 18 - line 23 page 23, line 3 - line 9 page 23, line 21 - line 26 page 30, line 10 - line 30 page 31, line 11 - line 14 page 32, line 2 - line 29 page 34, line 31 -page 35, line 7 page 36, line 15 -page 37, last line figures 1,2 --- -/--	1,3-10, 12,15-18

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
12 November 1999	22/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Thomas, R.M.

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INTERNATIONAL SEARCH REPORT

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PCT/GB 99/02597

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	column 12, last paragraph -column 13, line 20	13

X	EP 0 442 295 A (BOC) 21 August 1991 (1991-08-21) abstract page 6, line 18 - line 22 page 6, line 46 - line 55 page 8, line 6 - line 13 page 8, line 50 - line 53 page 9, line 56 - line 57 page 10, line 55 -page 11, line 1	1,3,10, 17

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